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Distribution of Zn isotopes during Alzheimer's disease

F. Moynier[1,2*], J. Foriel[3], A.S. Shaw[4], M. Le Borgne[4,5]

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Abstract

Alzheimer's disease is associated with abnormal homeostasis of Zn, because of deposits of Zn-rich amyloid-β fibrils. This connection between Zn and brain aging provides a possibility to use changes in Zn homeostasis to study the evolution of the disease. Here, we studied the evolution of the Zn isotopic composition of brain, serum and red blood cells from APPswe/PSEN1dE9 transgenic mice, which develop Alzheimer's-like disease (including Aβ deposition starting after 6 months), in comparison to wild type controls. We found that wild type brains become progressively enriched in the lighter isotopes of Zn between 6 and 12 months. We interpret this enrichment in terms of changes in Zn speciation in the cytoplasm, where isotopically heavy unbound Zn^{2+} is bound to glutamate released by presynaptic vesicles of neurons. Brains from APPswe/PSEN1dE9 mice were enriched in the heavier isotopes of Zn when compared to wild type suggesting an increase in Zn content of the brain associated with Aβ plaques where Zn binds preferentially to isotopically heavy amino acids such as histidine and glutamate.

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Letter

Alzheimer's disease (AD) is the most common type of dementia and one of the main causes of death in high-income countries (GBD 2013 Mortality and Causes of Death Collaborators, 2015). The main physiological hallmarks of AD are the formation of senile plaques by extracellular deposits of amyloid β (Aβ) fibrils. In addition, the homeostasis of metals, such as Zn, is dysregulated in AD patients (*e.g.*, Baum *et al.*, 2010; Szewczyk, 2013). While the full role of Zn in AD is not totally clear yet, there is much evidence that Zn is involved in the formation of Aβ leading to an enrichment of Zn in the neocortex of AD patients (*e.g.*, Miller *et al.*, 2006; Religa *et al.*, 2006).

The dysregulation of Zn homeostasis associated with AD may be utilised as a new diagnosis tool. Alterations in the concentration of Zn in the AD brain may be propagated to other body organs and fluids via red blood cells (RBCs) and/or serum. Following this approach, several studies have compared the Zn concentration in the serum of control and AD patients with inconsistent results (Baum *et al.*, 2010). Elemental abundance variations in the serum may be subjected to many factors unrelated to AD, such as intestinal absorption of Zn (Vasto *et al.*, 2007), which potentially explain the result inconsistencies.

The stable isotopic composition of Zn naturally varies between body organs (Balter *et al.*, 2010, 2013; Moynier *et al.*, 2013; Jaouen *et al.*, 2016). In stable isotope geochemistry, isotope ratios are usually reported as a relative deviation from a standard. In the case of Zn, all the data are reported using the $\delta^x Zn$ defined as:

$$\delta^x Zn = \left[\frac{\left(\frac{^x Zn}{^{64} Zn} \right)_{sample}}{\left(\frac{^x Zn}{^{64} Zn} \right)_{JMC-3-0749\,L}} - 1 \right] \times 1000$$

where x = 66 or 68. The reference material used is the Zn "Lyon" standard JMC 3-0749 L (*e.g.*, see Moynier *et al.*, 2017 for review).

In particular, RBCs are enriched by ~0.5 ‰ (for $\delta^{66}Zn$) when compared to serum, and up to ~1 ‰ when compared to the brain (Balter *et al.*, 2010, 2013; Moynier *et al.*, 2013). Differences in isotope ratio were not altered by gender, genetic background or age (up to 16 weeks) in multiple organs of mice (Moynier *et al.*, 2013). This difference between organs is explained by isotope fractionation at equilibrium between different bonding environments of Zn where isotopically light Zn is enriched in cysteine-rich proteins in the brain (metallothionein) and isotopically heavy Zn enriched in histidine-rich proteins in RBCs (carbonic anhydrase) (Moynier *et al.*, 2013). Since the AD deregulates the Zn homeostasis, we reasoned that the ~0.5 ‰ Zn isotopic difference between serum and brain of normal mice could be used to search for modifications of the isotopic balance during the development of the AD. For example, Zn is principally bound to histidine in amyloid-beta that are formed during the AD (Faller and Hureau, 2009), which should enrich the AD brains in isotopically heavy Zn compared to controls and possibly deplete the serum in the lighter isotopes.

1. Institut de Physique du Globe de Paris, Université Sorbonne Paris Cité, Université Paris Diderot, 1 rue Jussieu, 75238 Paris Cedex 05, France
2. Institut Universitaire de France, 75005 Paris, France
* Corresponding author (email: moynier@ipgp.fr)
3. Earth Life Science Institute, Tokyo Institute of Technology, 2-12-1-IE-1 Ookayama, Meguro, Tokyo 152-8551, Japan
4. Department of Pathology and Immunology and Howard Hughes Medical Institute, Washington University School of Medicine in St. Louis, USA
5. Unité 1148, Institut National de la Santé et de la Recherche Médicale (INSERM), Hôpital Xavier Bichat, Paris, France; Université Paris Diderot, Sorbonne Paris Cité, Paris, France; Département Hospitalo-Universitaire DHU FIRE, Paris, France

Here we tested whether the distribution of Zn isotopes is altered in mice with late stage AD and determined how early disease anomalies in Zn isotopes could be detected. We present the Zn isotopic composition of transgenic APPswe/PSEN1dE9 and wild type (WT) controls for which we have collected the brain, RBCs and serum at the early stage (6 months) and late stage of AD (9 and 12 months) (Jankowsky *et al.*, 2004; Garcia-Alloza *et al.*, 2006) to assess how AD affects the balance of zinc isotopes in the brain.

Brains are enriched in lighter isotopes compared to blood. The method, sample descriptions and Zn isotopic data for 128 samples are reported in the Supplementary Information. The average of the different group of samples sorted by ages are reported in Table S-2. All the mice were fed the same diet (water and dry food) from birth and were housed in the same animal facility as our previous study (Moynier *et al.*, 2013); we can therefore directly compare the isotopic composition of the two studies. Assuming that the Zn isotopic composition of the mouse food does not vary between different batches, we can use the value from our previous study: $\delta^{66}Zn = 0.30 \pm 0.10$ ‰. On average, the RBCs were isotopically heavier ($\delta^{66}Zn$ ~+1 ‰) than serum ($\delta^{66}Zn$ ~+0.5 ‰) and brains ($-0.5 < \delta^{66}Zn < 0$ ‰) (Fig. 1). These results are in excellent agreement with what we previously reported (Moynier *et al.*, 2013).

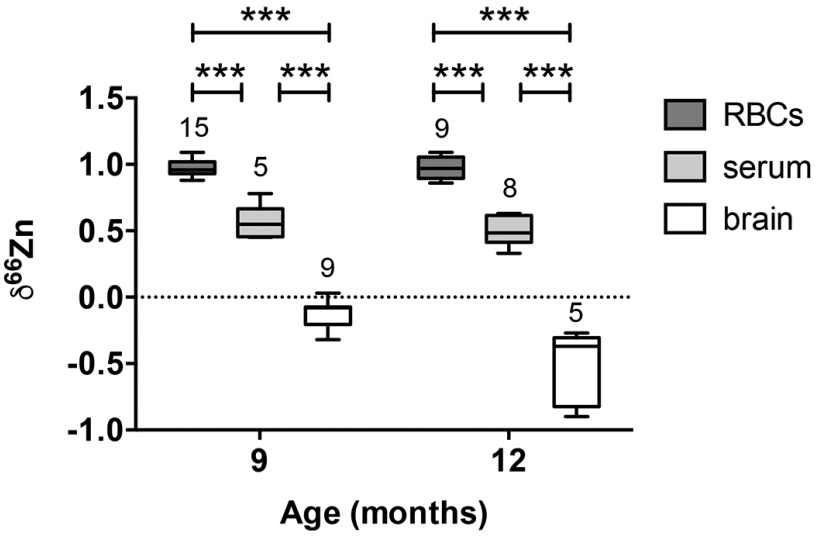

Figure 1 $\delta^{66}Zn$ of brains, serum and RBCs of WT mice over time. Boxes extend from the 25th and 75th percentile, the line in the middle of the box represents the median, and the whiskers show the minimum and the maximum. Two-way ANOVA was performed to compare groups (F value: 11.95, $p < 0.0001$), followed by Sidak's multiple comparison test to compare the means between organs for each time point (*** indicates statistical difference, $p < 0.001$).

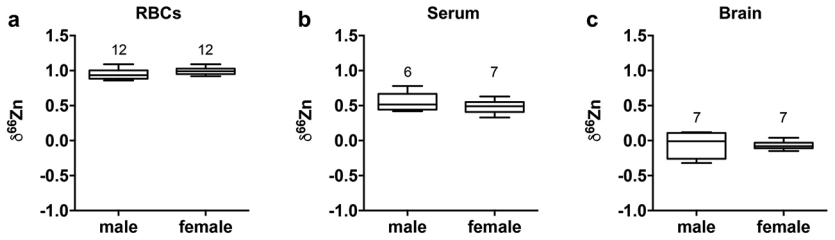

Figure 2 Effect of the sex on $\delta^{66}Zn$. Data show pooled results from 9- and 12-month-old WT mice for **(a)** RBCs and **(b)** serum, and **(c)** 6- and 9-month-old WT mice for brain. Student's t-test showed no significant differences in any organ between males and females.

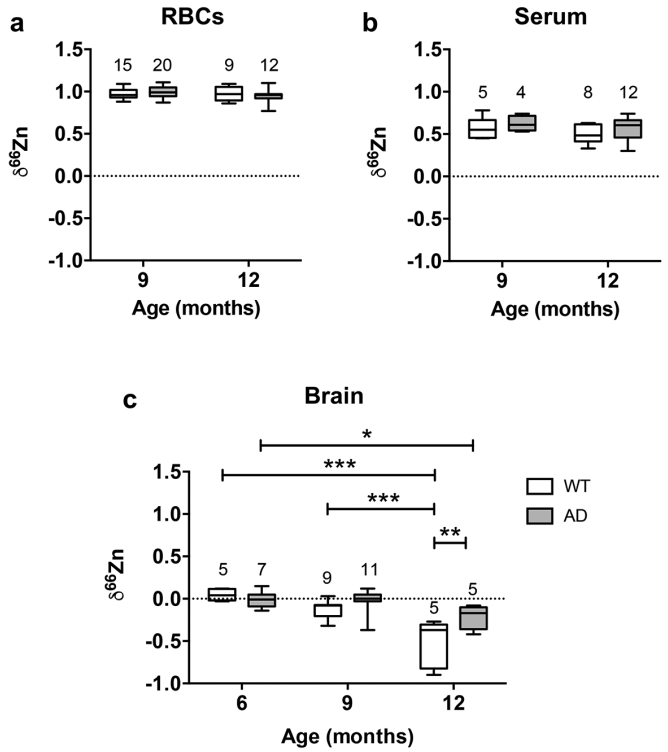

Figure 3 Effect of Alzheimer's disease on $\delta^{66}Zn$. Data show results from WT and AD mice for **(a)** RBCs, **(b)** serum and **(c)** brain. Two-way ANOVA showed no significant effect of age or genotype for RBCs ($F = 1.953$, $p = 0.17$; a) or serum ($F = 0.008$, $p = 0.92$; b), and showed significant effect of age and genotype ($F = 4.288$, $p = 0.0214$) for brain (c). Sidak's multiple comparison tests were run to compare groups for brain samples: asterisks indicate significant difference between groups (*: $p < 0.05$; **: $p < 0.01$; *** $p < 0.001$).

Alzheimer's disease does not affect the isotopic ratio in the blood. The values obtained for RBCs were extremely homogeneous ($\delta^{66}Zn$ = 0.97 ± 0.07 ‰, n = 24) for WT, with no dependence on sex (Fig. 2a) or age (Fig. 1, $p > 0.05$ with Sidak's multiple comparison test). The values for the serum were slightly more variable ($\delta^{66}Zn$ = 0.52 ± 0.12 ‰ for WT, n = 13), with no effect of sex (Fig. 2b), or age (Fig. 1; $p > 0.05$ with Sidak's multiple comparison test).

Finally, there was no effect of the genotype on the isotopic composition of the blood compartments, as there was no significant difference between AD mice and WT mice in RBCs (Fig. 3a) and serum (Fig. 3b).

Alzheimer's disease delays brain enrichment in lighter isotopes over time. The $\delta^{66}Zn$ of the brain showed the largest range of variation among the organs, with an average of -0.19 ± 0.27 ‰; n = 19 for WT mice. Sex had no significant impact on the isotopic composition of the brain (Fig. 2c). But the variations were correlated with mouse age (Figs. 1 and 3c) with a decrease in the $\delta^{66}Zn$ in comparing 6-month-old to 12-month-old mice.

Even if brains also got enriched in the lighter isotopes over time in AD mice, they clearly displayed a heavier Zn isotopic ratio than WT mice in 12-month-old animals ($p < 0.01$, Sidak's multiple comparison test) and therefore AD brains are isotopically different from WT brains. These data show that AD has an effect on the Zn isotopic ratio in the brain.

Effect of aging on the Zn isotopic composition of organs and its implication for the dysregulation of the homeostasis of Zn. The youngest mice that we analysed in this study are 6 months old, and have Zn isotopic composition of the brains (0.05 ± 0.07 ‰; n = 5) identical to the mice measured in previous studies (-0.03 ± 0.08 ‰; n = 14, Moynier *et al.*, 2013). This suggests that up to 6 months there is no change in the Zn isotopic composition of the brain. However, brains of 9- and 12-month-old WT and AD mice had enrichment of the lighter isotopes (Fig. 3c). This enrichment in the lighter isotopes can potentially be used to understand what processes associated with Zn are changing with normal aging.

The changes in $\delta^{66}Zn$ of brains with age must be related to the deterioration of brain cells. It is well known that aging brains display several changes, such as a decrease of the number of neurons as well as a decline in the cell-to-cell communications due to a deterioration of the synaptic contact zone (Casoli *et al.*, 1996; Bertoni-Freddari *et al.*, 2008). In the brain, Zn is mostly distributed within three reservoirs in three different species (Szewczyk, 2013): 1) sequestered in the presynaptic vesicles of neurons co-located with glutamate (Frederickson, 1989; Frederickson *et al.*, 2000); 2) bound to intra-cellular proteins such as metallothioneins (MT) (Krezel *et al.*, 2007), ZIP and membranous transporter proteins (ZnT) (Huang and Tepaamorndech, 2013); and 3) unbound ions in the cytoplasm as hydrated Zn^{2+} (Frederickson *et al.*, 2000).

The exact behaviour and role of Zn during normal brain aging is still unclear: some studies showed that unbound Zn^{2+} increases (Bertoni-Freddari *et al.*, 2008) whereas other studies suggest that it decreases (Mocchegiani *et al.*, 2005). Possible explanations for the decrease of free Zn would be the excessive pre-synaptic release of glutamate (Saito *et al.*, 2000) or the increased expression of proteins such as MT (Mocchegiani *et al.*, 2005) that would bind to Zn^{2+}. The enrichment in the light isotopes of Zn with age provides a new way to test the behaviour of Zn during aging. Since total Zn remains constant in the brain through normal aging (Rahil-Khazen *et al.*, 2002), the changes in isotopic composition must be related to changes in Zn speciation in the brain. The bonding environment to be considered is that Zn is bound to cysteines in MT, histidines in ZnT and ZIP proteins, glutamates in pre-synaptic vesicles, and unbounded hydrated Zn in the cytoplasm. If we consider a four-fold coordination, the logarithm of the reduced partition ($\ln\beta$) for the $^{66}Zn/^{64}Zn$ ratio decreases from Zn-histidine ($\ln\beta$ ~3.7), Zn-glutamate and $Zn(H_2O)_4^{2+}$ ($\ln\beta$ ~3.6), to Zn-cysteine ($\ln\beta$ ~3.1) (Fujii *et al.*, 2014). The $\ln\beta$ can be directly compared to each other to estimate the variations in the $\delta^{66}Zn$, *i.e.* that $\delta^{66}Zn(\text{histidine}) - \delta^{66}Zn(\text{cysteine}) = \ln\beta(\text{histidine}) - \ln\beta(\text{cysteine})$ at ~0.6 ‰.

The decrease of the $\delta^{66}Zn$ with age suggests an increase of the Zn pool with the lightest isotopic composition. The behaviour of Zn isotopes is consistent with a decrease of isotopically heavy Zn^{2+}. It is however inconsistent with the binding of free Zn^{2+} to glutamate-Zn (Saito *et al.*, 2000) because both species have similar $\ln\beta$. Therefore, the depletion in Zn^{2+} is coupled with an increase in isotopically light Zn bound preferentially to proteins such as MT (Mocchegiani *et al.*, 2005).

Contrary to the brains, the Zn isotopic composition of RBCs and serum does not change with age (Figs. 1 and 3). This suggests that $\delta^{66}Zn$ of serum and RBCs could be used as tracers of changes in Zn metabolism with no need to correct for the age of the patients.

Effect of Alzheimer's disease on the Zn isotopic composition of the brain. Alzheimer's disease mice start to develop Aβ plaques from 6 months with abundant plaques in the hippocampus and cortex by 9 months (Jankowsky *et al.*, 2004) and with increased plaque burden until 12 months (Garcia-Alloza *et al.*, 2006). Therefore the development of Aβ plaques in AD mice is coincident with the isotopic fractionation of Zn due to normal aging. While this result complicates the interpretations of the AD mice data, it is clear that there is an isotopic difference between the WT and the AD mice, with the latter heavier than the former in older mice (Fig. 3c). This enrichment in the heavier isotopes of Zn of AD mice is consistent with Aβ plaques enriched in the heavier isotopes of Zn compared to normal brain. Zinc is principally bound to histidine in Aβ (Faller and Hureau, 2009), which is isotopically heavier than Zn-MT (Moynier *et al.*, 2013), the main Zn carrier in normal brains.

The Zn concentration of brains was found to double in AD patients (Religa *et al.*, 2006), in agreement with many studies that found enrichment of Zn in different brain regions such as hippocampus (Deibel *et al.*, 1996), or amygdala

(Deibel *et al.*, 1996; Lovell *et al.*, 1998). Zinc in Aβ plaques is widely considered to bind to four ligands including three histidine residues in the N-terminal hydrophilic region Aβ16 of the Aβ peptide (Faller and Hureau, 2009; Pithadia and Lim, 2012), with a fourth possible binding site being either aspartate or glutamate (Faller and Hureau, 2009). While no *ab initio* calculations exist for aspartate, Zn bound to either the N-terminal amine or to O in the carboxylate function of aspartate (Faller and Hureau, 2009) should enrich the heavier isotopes of Zn (Moynier *et al.*, 2013). Therefore, we can consider that Aβ δ^{66}Zn is closer to histidine and glutamate. If we consider that 1) there is the same isotopic difference between Zn-Aβ and Zn-MT in normal brain as between Zn-histidine (or glutamate) and Zn-cysteine at ~0.6 ‰ and 2) that AD brain has twice as much Zn as normal aging brain, then AD brains are 0.3 ‰ heavier than normal brains, similar to our observations.

Our study substantiates the potential of Zn isotopes as a tool to understand Zn homeostasis in living organisms. δ^{66}Zn of brains change with time suggesting a change in the speciation of Zn, with a decrease in the unbound Zn^{2+} associated with an increase in Zn-MT. δ^{66}Zn of the brain is altered in AD, with a progressive enrichment in the heavier isotopes of Zn associated with an increase in Aβ plaques. These results can be explained by an increase in Zn content of the brain associated with Aβ plaques where isotopically heavy Zn binds preferentially to histidine and glutamate.

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Additional Information

Supplementary Information accompanies this letter at www.geochemicalperspectivesletters.org/article1717

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